Phospholipase A_2 Is Involved in the Mechanism of Activation of Neutrophils by Polychlorinated Biphenyls

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Aroclor 1242, a mixture of polychlorinated biphenyls (PCBs), activates neutrophils to produce superoxide anion (O_2^-) by a mechanism that involves phospholipase C-dependent hydrolysis of membrane phosphoinositides; however, subsequent signal transduction mechanisms are unknown. We undertook this study to determine whether phospholipase A_2-dependent release of arachidonic acid is involved in PCB-induced O_2^- production. We measured O_2^- production *in vitro* in glycogen-elicited, rat neutrophils in the presence and absence of the inhibitors of phospholipase A_2: quinacrine, 4-bromophenacyl bromide (BPB), and manoalide. All three agents significantly decreased the amount of O_2^- detected during stimulation of neutrophils with Aroclor 1242. Similar inhibition occurred when neutrophils were activated with the classical stimuli, formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate. The effects of BPB and manoalide were not a result of cytotoxicity or other nonspecific effects, although data suggest that quinacrine is an O_2^- scavenger. Significant release of ^{3}H-arachidonic acid preceded O_2^- production in neutrophils stimulated with Aroclor 1242 or fMLP. Manoalide, at a concentration that abolished O_2^- production, also inhibited the release of ^{3}H-arachidonate. Aspirin, zileuton, or WEB 2086 did not affect Aroclor 1242-induced O_2^- production, suggesting that eicosanoids and platelet-activating factor are not needed for neutrophil activation by PCBs. Activation of phospholipase A_2 and O_2^- production do not appear to involve the Ah receptor because a congener with low affinity, but not one with high affinity for this receptor, stimulated the release of arachidonic acid and O_2^-. These data suggest that Aroclor 1242 stimulates neutrophils to produce O_2^- by a mechanism that involves phospholipase A_2-dependent release of arachidonic acid. *Key words*: arachidonic acid, 4-bromophenacyl bromide, manoalide, PCBs, quinacrine, signal transduction, superoxide anion. *Environ Health Perspect* 104:52–58 (1996)

Polychlorinated biphenyls (PCBs) are a class of industrial chemicals that persist in the environment. In laboratory animals PCBs cause a variety of biological effects including cancer, immunotoxicity, neurotoxicity, and hepatotoxicity as well as dermatitis, edema, induction of cytochrome P450, and increased mixed-function oxidase activities. Although many of these effects have been related to activity of the Ah receptor, recent studies suggest that mechanisms independent of this receptor may also be important (*1–3*). For example, investigations concerning the neurotoxicity of PCBs suggest that *ortho*-substituted congeners, which have little affinity for the Ah receptor, cause greater decreases in neurotransmitter levels (*1*) and perturbations of Ca^{2+} homeostasis in neural cells (*3,4*) than planar congeners, which bind the Ah receptor with high affinity.

Studies in our laboratory indicate similar results in neutrophils. When exposed *in vitro* to Aroclor 1242, a PCB mixture, neutrophils are activated to undergo degranulation and to produce superoxide anion (O_2^-). These effects do not appear to involve Ah receptor activity since similar results are observed with an *ortho*-substituted congener, 2,2′,4,4′-tetrachlorobiphenyl (2,2′,4,4′-TCB), whereas a planar congener, 3,3′,4,4′-tetrachlorobiphenyl (3,3′,4,4′-TCB), does not alter neutrophil function (*5*). Furthermore, the activation of neutrophils by PCBs appears to involve mechanisms similar to those reported by Kodavanti et al. (*4,5*) for neural cells, namely, the activation of phospholipase C, the production of inositol 1,4,5-trisphosphate (IP_3), and the subsequent mobilization of intracellular calcium (*6*). Activation of phospholipase C appears to be important in PCB-induced stimulation of neutrophils because the concentration–response relationship is similar for production of inositol phosphates and neutrophil activation, and IP_3 accumulation precedes the onset of O_2^- production in neutrophils exposed to PCBs. Furthermore, activity of this enzyme parallels production of O_2^-; i.e., 2,2′,4,4′-TCB causes the accumulation of inositol phosphates but 3,3′,4,4′-TCB does not (*6*). Finally, 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoic acid, an inhibitor of mobilization of intracellular calcium, significantly attenuates PCB-induced neutrophil activation, suggesting that IP_3-induced mobilization of intracellular calcium plays an important role in the activation of neutrophils by PCBs (*7*). These results are consistent with the hypothesis that PCBs activate neutrophils by a mechanism that involves phospholipase C. Little is known, however, about subsequent signaling pathways involved in the activation of neutrophils by PCBs.

Phospholipase C appears to play an important role in neutrophil activation in response to several physiologic activators of neutrophil function, including the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) (*8,9*), opsonized zymosan, and antigen-antibody complexes (*10*). Several enzymes are activated subsequent to the increase in phospholipase C activity, including phospholipases A_2 and D, as well as protein kinase C (*11*). Activation of these enzymes is important in regulation, initiation, and/or maintenance of neutrophil activation. For example, inhibitors of phospholipase A_2 such as quinacrine and 4-bromophenacyl bromide (BPB) abolish O_2^- production in response to fMLP and opsonized zymosan, as well as to phorbol myristate acetate (PMA) (*12–14*), suggesting that activity of phospholipase A_2 is required for activation of the NADPH oxidase responsible for reduction of O_2 to O_2^-. Because early signal transduction events are similar in neutrophils exposed to PCBs and other stimuli such as fMLP, it is possible that subsequent signal transduction mechanisms are also similar. Therefore, the purpose of the present study was to determine whether PCBs activate neutrophils by a mechanism that involves phospholipase A_2 and consequent release of arachidonic acid.

Materials and Methods

Chemicals. Aroclor 1242, 2,2′,4,4′-TCB, 3,3′,4,4′-TCB, and 3,3′4,4′,5-pentachlorobiphenyl (PeCB) were obtained from ChemService (West Chester, Pennsylvania). The congeners were reported by the manufacturer to be greater than 99% pure. fMLP, PMA, cytochalasin B, cytochrome C, superoxide dismutase (SOD), quinacrine, BPB, xanthine, and xanthine oxidase were

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obtained from Sigma Chemical Company (St. Louis, Missouri). WEB 2086 was obtained from Boehringer Ingelheim (Ridgefield, Connecticut) and zileuton from Abbott Laboratories (Chicago, Illinois). Manoalide was purchased from Biomol (Plymouth Meeting, Pennsylvania), and ^{3}H-arachidonate was obtained from DuPont (Boston, Massachusetts). For all experiments Aroclor 1242 was dissolved in methanol, and the congeners were dissolved in dimethylformamide at a concentration of 10 mg/ml. Neutrophils received 1 µl/ml of this stock solution, and control cells received 1 µl of the appropriate vehicle. fMLP, PMA, and cytochalasin B were dissolved in DMSO and diluted so that the final concentration of DMSO was less than 1%. Manoalide and BPB were suspended in ethanol so that the final concentration of ethanol to which neutrophils were exposed was less than 1%. Quinacrine was suspended in Hanks' balanced salt solution (HBSS).

Neutrophil isolation. We obtained glycogen-elicited neutrophils from the peritoneal cavities of one to two male, Sprague-Dawley, retired breeder rats per experiment (Charles River Laboratories, Portage, Michigan) as described previously (*5*). Briefly, rats were anesthetized with diethyl ether and 30 ml 1% glycogen was injected intraperitoneally. After 4 hr the rats were anesthetized again and killed by decapitation. The peritoneum was washed with 30 ml of heparinized (1 U/ml), 0.1 M phosphate-buffered saline (PBS). The solution obtained from the peritoneum was collected, filtered through gauze, and centrifuged at 500*g* for 7 min. Contaminating red blood cells were lysed with 15 ml of 0.15 M NH_4Cl, and neutrophils were suspended to a final volume of 50 ml with PBS and centrifuged for 7 min at 300*g*. Cells were washed once with PBS and resuspended in HBSS.

Generation of O_2^-. We measured O_2^- production by neutrophils (2×10^6 cells/ml) in response to Aroclor 1242, 2,2′,4,4′-TCB, 3,3′,4,4′-TCB, 3,3′,4,4′5-PCB, PMA, and fMLP. In these and all experiments with fMLP, neutrophils were pretreated with cytochalasin B (4 µM) for 5 min because fMLP does not cause significant O_2^- production in rat neutrophils in the absence of cytochalasin B (*6,15*).

We measured O_2^- production in the presence and absence of the inhibitors of phospholipase A_2, BPB (1–100 µM), quinacrine (1–200 µM), and manoalide (0.1–10 µM). Neutrophils were preincubated with BPB or quinacrine for 3 min or with manoalide for 20 min before addition of stimuli. To test the role of metabolites of arachidonic acid in neutrophil activation, O_2^- production was measured in stimulated neutrophils in the presence and absence of aspirin or zileuton, inhibitors of cyclooxygenase or 5-lipoxygenase, respectively. We used the receptor antagonist for platelet-activating factor (PAF), WEB 2086, to examine the potential involvement of PAF. Neutrophils were incubated with aspirin or WEB 2086 for 30 min or with zileuton for 15 min before addition of stimuli. In all experiments, cumulative O_2^- production in response to stimulation was measured over 20 min.

Detection of O_2^-. We measured O_2^- generated by monitoring the reduction of cytochrome C (10 mg/ml) in the presence and absence of SOD (*16*). For every sample, two tubes were incubated, one to which SOD (840 U/ml) was added before stimulation and one to which SOD (840 U/ml) was added at the end of incubation. The amount of cytochrome C reduced was estimated from the difference in absorbance (550 nm) between the cell-free supernatant in the two tubes using an extinction coefficient of 18.5 cm^{-1} mM^{-1}.

Phospholipase A_2 inhibitors could decrease the amount of O_2^- detected by mechanisms unrelated to inhibition of production of O_2^- by neutrophils. To test whether any apparent decrease in O_2^- production by inhibitors of phospholipase A_2 was due to scavenging of oxygen radicals, we measured O_2^- generated during oxidation of xanthine by xanthine oxidase in a cell-free system in the presence and absence of the compounds. Cytochrome C was incubated for 5 min at 37°C with xanthine (9 mg/ml) and xanthine oxidase (2.5×10^{-4} U/ml) in the presence or absence of quinacrine, BPB, and manoalide. We determined the amount of O_2^- generated by xanthine and xanthine oxidase by the difference in absorbance (550 nm) between tubes containing both xanthine and xanthine oxidase and tubes containing xanthine alone.

In addition, absorbance of reduced cytochrome C was measured in the presence or absence of phospholipase A_2 inhibitors to ensure that apparent inhibition of neutrophil-generated O_2^- was not a result of quenching of absorbance by these compounds. Cytochrome C was reduced by O_2^- generated from neutrophils exposed to Aroclor 1242 as described above, and absorbance at 550 nm was measured in the cell-free supernatant fluids after the addition of quinacrine, manoalide, BPB, or the appropriate vehicle. There were no differences in absorbance values in the presence and absence of inhibitors of phospholipase A_2.

Determination of cytotoxicity. Cytotoxicity was determined by release of the cytosolic enzyme lactate dehydrogenase (LDH). Neutrophils were incubated with manoalide, BPB, or quinacrine as described above, and LDH activity was determined in the cell-free supernatant fluids as described by Bergmeyer and Bernt (*17*). A separate aliquot of neutrophils was lysed with Triton-X and sonication, and total LDH activity was determined in the cell-free supernatant fluid of this lysate so that cytotoxicity could be expressed as the percentage of total LDH released.

Labeling of neutrophils with ^{3}H-arachidonic acid. Neutrophils (10^7/ml) were suspended in Mg^{2+}-free and Ca^{2+}-free HBSS containing 0.1% bovine serum albumin and incubated in the presence of 0.5 µCi/ml ^{3}H-arachidonic acid (specific activity = 180–240 Ci/mM) for 2 hr gently shaking at 37°C. At the end of the labeling period, neutrophils were washed 2 times with Mg^{2+}-free and Ca^{2+}-free HBSS and resuspended in HBSS containing albumin, Ca^{2+} and Mg^{2+}. The cell count was adjusted so that the final concentration of neutrophils was 2×10^6/ml. The incorporation of ^{3}H-arachidonate was routinely greater than 80% of the total radioactivity added to the cells.

Stimulated release of ^{3}H-arachidonate. Neutrophils labeled as described above were stimulated with either fMLP, PMA, Aroclor 1242, 2,2′,4,4′-TCB, 3,3′,4,4′-TCB, 3,3′,4,4′,5-PeCB, or the appropriate vehicle, and release of ^{3}H-arachidonate was measured at various times. In some experiments, we measured ^{3}H-arachidonate release in the presence and absence of manoalide (10 µM). At the end of each incubation, neutrophils were placed on ice and centrifuged at 0°C for 10 min. The cell-free supernatant fluids were transferred to vials containing scintillation cocktail (14 ml), and the total radioactivity in each sample was determined by liquid scintillation counting. To determine the total cellular uptake of ^{3}H-arachidonate, for each experiment total cellular radioactivity was measured in a 1-ml aliquot of washed cells (2×10^6 cells/ml).

Statistical analysis. Results are expressed as means ± standard error of the mean (SEM). Results for ^{3}H-arachidonate are expressed as percentage of total cellular radioactivity released into the medium. Data were analyzed by analysis of variance, and group means were compared using the Student-Newman-Keuls' test. We performed appropriate transformations on all data that did not follow a normal distribution (e.g., percent data). Neutrophils taken from a single rat or pooled from two rats were used to obtain data for a single replicate or *n*. For all studies, the criterion for statistical significance was $p \leq 0.05$.

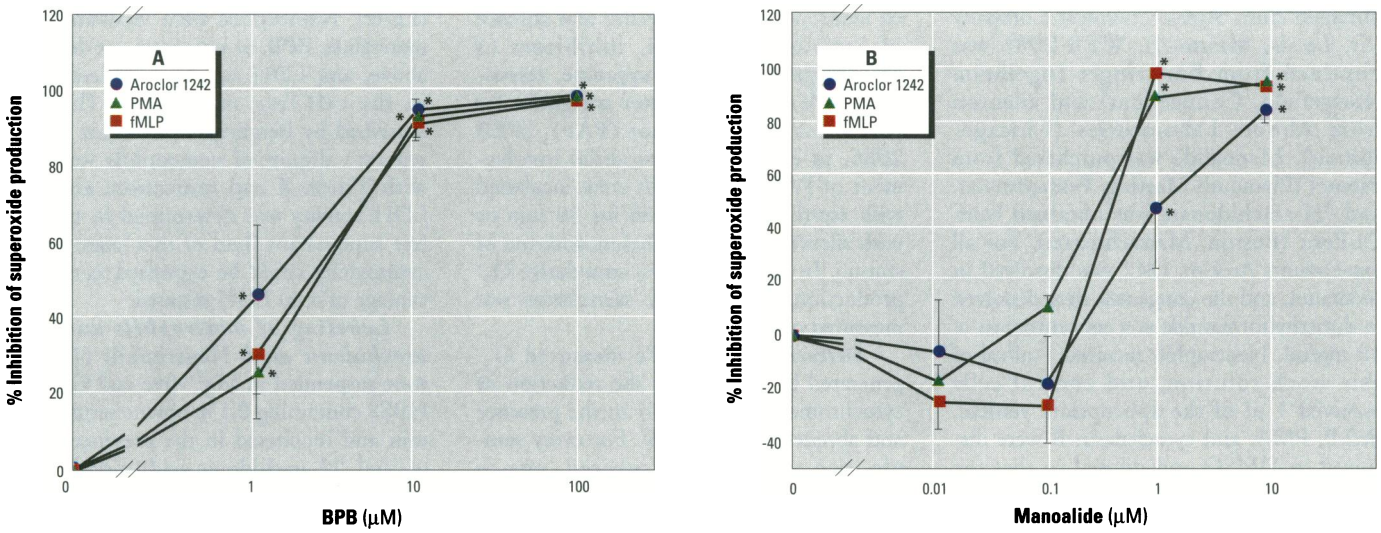

Figure 1. Percent inhibition of superoxide anion (O_2^-) generation by (A) 4-bromophenacyl bromide (BPB) and (B) manoalide. O_2^- production was measured in neutrophils as described in Materials and Methods. Neutrophils (2×10^6 cells/ml) were incubated at 37°C for 3 min in the presence of BPB or vehicle or for 20 min in the presence of manoalide or vehicle and then stimulated for 20 min with Aroclor 1242 (10 μg/ml), phorbol myristate acetate (PMA; 20 ng/ml) or formyl-methionyl-leucyl-phenylalanine (fMLP; 1 μM). Cytochalasin B (4 μM) was added 5 min before stimulation with fMLP. O_2^- production in response to Aroclor 1242, PMA, and fMLP was (A) 25.6 ± 5.8, 45.8 ± 14.5, and 19.6 ± 4.6 nmol/10^6 cells/20 min and (B) 13.8 ± 5.1, 21.3 ± 2.4, and 14.9 ± 3.9 nmol/10^6 cells/20 min. *Significantly different from respective value obtained in the absence of inhibitor; n = 4–5.

Results

No O_2^- was produced by unstimulated neutrophils either in the presence or absence of the phospholipase A_2 inhibitors BPB, manoalide, or quinacrine (data not shown). The effect of BPB on O_2^- production in neutrophils stimulated with Aroclor 1242, fMLP, and PMA is shown in Figure 1A. Cumulative O_2^- release in response to Aroclor 1242 was 25.6 ± 5.8 nmol/10^6 cells/20 min. Responses to PMA and fMLP were included so that effects of PCBs could be compared to those of other neutrophil stimuli with well-studied mechanisms of action. PMA and fMLP produced 45.8 ± 14.5 and 19.6 ± 4.6 nmol/10^6cells/20 min, respectively. O_2^- produced by all three agents was significantly different from unstimulated neutrophils. O_2^- generated in the presence of Aroclor 1242 was significantly inhibited by 1 μM BPB and abolished by 10 μM BPB (Fig. 1A). Similar inhibition of fMLP- and PMA-induced O_2^- production was observed.

Manoalide also inhibited O_2^- production in neutrophils exposed to Aroclor 1242, fMLP, and PMA; however, the concentration–response relation differed slightly in neutrophils exposed to fMLP and PMA compared to Aroclor 1242 (Fig. 1B). Inhibition of fMLP- and PMA-induced O_2^- production was complete in the presence of 1 μM manoalide, i.e., maximal, inhibition of O_2^- generation in the presence of Aroclor 1242 was achieved with 10 μM manoalide (Fig. 1B). Neither BPB nor manoalide was cytotoxic to neutrophils in the presence of stimuli (Fig. 2). In addition, neither of these phospholipase A_2 inhibitors scavenged O_2^- radicals at concentrations that inhibited neutrophil O_2^- production as evidenced by the inability of these agents to reduce the amount of O_2^- detected in the presence of xanthine and xanthine oxidase (Table 1). The quantity of O_2^- measured in neutrophils exposed to Aroclor 1242, fMLP, or PMA was also decreased in a concentration-dependent manner by quinacrine (Fig. 3); however, this compound inhibited the amount of O_2^- detected in the presence of xanthine and xanthine oxidase in a cell-free system (Table 1), suggesting that quinacrine scavenges O_2^- radicals.

The effect of BPB and manoalide to inhibit O_2^- production by Aroclor 1242-stimulated neutrophils suggested that Aroclor 1242 activates phospholipase A_2. A consequence of activation of this enzyme is liberation of arachidonic acid from membrane phospholipids. Accordingly, we examined the kinetics of ^{3}H-arachidonate release in response to Aroclor 1242. Experiments were performed in the presence of albumin to inhibit the metabolism and reacylation of arachidonic acid; therefore, the data represent the cumulative release of arachidonic acid. Little radiolabeled arachidonate was released from neutrophils in the absence of activation (Fig. 4). Significant release of ^{3}H-arachidonate was observed within 5 min from neutrophils exposed to Aroclor 1242, and no further release was observed after this time (Fig. 4A). ^{3}H-arachidonate release in response to fMLP occurred within 2 min,

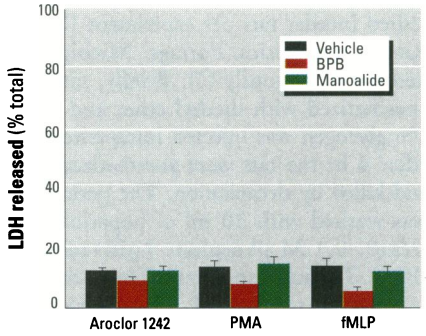

Figure 2. Lack of cytotoxicity of manoalide and 4-bromophenacyl bromide (BPB). Neutrophils were exposed to Aroclor 1242, phorbol myristate acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence or absence of manoalide (10 μM) or BPB (100 μM) as described in the legend to Figure 1, and cytotoxicity was assessed from release of the cytosolic enzyme lactate dehydrogenase (LDH) as described in Materials and Methods. Percent release of LDH from unstimulated neutrophils was 7.5 ± 1.6%; n = 3. There were no significant differences between groups.

with no further release thereafter (Fig. 4B). The magnitude of release of ^{3}H-arachidonate was greater in neutrophils exposed to Aroclor 1242 when compared to fMLP (Fig. 4A, B). In neutrophils exposed to PMA, a small but statistically significant release of ^{3}H-arachidonate was observed, but not until 20 min after stimulation (Fig. 4C).

Because release of arachidonic acid can occur by mechanisms independent of phospholipase A_2 (*18*), we investigated the effect of manoalide on PCB-, PMA-, and

fMLP-induced ^{3}H-arachidonate release. As can be seen in Figure 5A, manoalide, at a concentration which abolished O_2^- production by all three agents (Fig. 2B), significantly inhibited the release of ^{3}H-arachidonate in neutrophils stimulated with Aroclor 1242. Similar results were observed in neutrophils stimulated with fMLP (Fig. 5B). PMA induced a small increase in arachidonic acid release that was significant in the absence, but not the presence of manoalide, and these values were not significantly different from each other (Fig. 5C). Similar results were obtained with BPB (data not shown).

The release of ^{3}H-arachidonate and the production of O_2^- by neutrophils in response to stimulation with Aroclor 1242 were concentration dependent (Fig. 6A). Likewise, exposure to 2,2′,4,4′-TCB caused generation of O_2^- and release of ^{3}H-arachidonate in a concentration-dependent manner (Fig. 6B), whereas 3,3′,4,4′-TCB failed to elicit either response in neutrophils (Fig. 6C). A small but significant release of ^{3}H-arachidonate was observed in neutrophils exposed to 3,3′,4,4′,5-PeCB; however, this congener did not cause significant production of O_2^- (Fig. 6D).

Arachidonic acid is metabolized in the neutrophil by cyclooxygenase and 5-lipoxygenase to products that can alter neutrophil function (*19*). To test whether arachidonic acid metabolites were involved in activation of the NADPH oxidase, we measured O_2^- production in stimulated neutrophils in the presence and absence of the inhibitor of cyclooxygenase, aspirin, or the inhibitor of 5-lipoxygenase, zileuton (Table 2). O_2^- production in neutrophils exposed to Aroclor 1242 was not affected by these inhibitors at concentrations that have been shown to attenuate eicosanoid production (*20*). Similarly, no effect of these inhibitors on fMLP- or PMA-induced O_2^- production was observed. PAF is a product of phospholipase A_2, which has been suggested to be involved in the generation of O_2^- in rabbit neutrophils (*21*); therefore, experiments with the PAF receptor antagonist WEB 2086 were performed to test for any involvement of this product. WEB 2086, at concentrations which significantly attenuate PAF-induced responses (*22*), did not alter the generation of O_2^- by Aroclor 1242, PMA, or fMLP (Table 2).

Table 1. Detection of O_2^- generated from xanthine and xanthine oxidase[a]

Phospholipase A_2 inhibitor	Concentration (μM)	O_2^- detected (nmol/ml)
Vehicle	0	33 ± 6.2
Quinacrine	100	4.7 ± 2.7*
	200	6.0 ± 3.6*
Manoalide	10	27 ± 0.1
4-Bromophenacyl bromide	100	29 ± 4.8

[a]The effect of inhibitors of phospholipase A_2 on O_2^- generated from a mixture of xanthine (9 mg/ml) and xanthine oxidase (2.5×10^{-4} U/ml) was measured in a cell-free system. O_2^- generated was assessed as described in Materials and Methods. The amount of O_2^- detected was significantly decreased in the presence of quinacrine, but not manoalide or 4-bromophenacyl bromide. *Significantly different from value obtained in the presence of vehicle; $n = 3$.

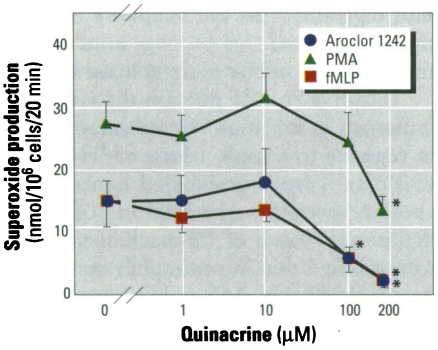

Figure 3. Quinacrine induced-inhibition of O_2^- generation. Experiments were performed as described in the legend to Figure 1, except that neutrophils were incubated at 37°C for 3 min in the presence or absence of quinacrine prior to stimulation for 20 min. *Significantly different from respective value obtained in the absence of quinacrine; $n = 4$–5.

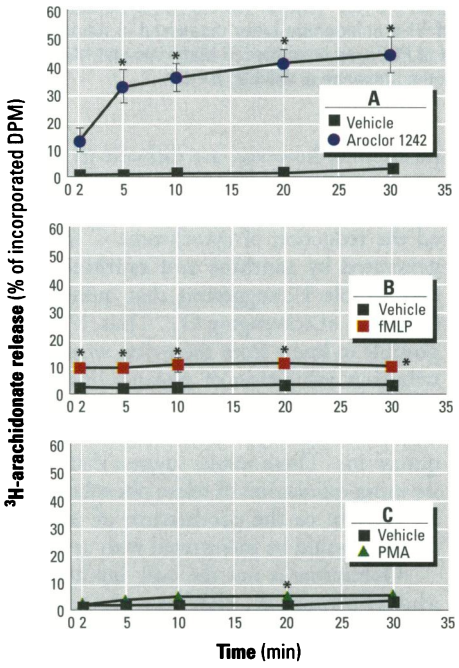

Figure 4. Time course of cumulative release of ^{3}H-arachidonic acid from neutrophils. Neutrophils were labeled as described in Materials and Methods and incubated in the presence and absence of (A) Aroclor 1242, (B) formyl-methionyl-leucyl-phenylalanine (fMLP), or (C) phorbol myristate acetate (PMA) as described in the legend to Figure 1. Release of ^{3}H-arachidonic acid into the extracellular medium was determined as described in Materials and Methods. Data are expressed as percent of total cellular radioactivity. *Statistically different from respective results obtained in the presence of vehicle; $n = 3$–4.

Discussion

We have demonstrated previously that the PCB mixture Aroclor 1242 activates isolated rat neutrophils to produce O_2^- (*5*). In the present study, Aroclor 1242 stimulated the release of arachidonic acid by a mechanism involving phospholipase A_2. The release of arachidonic acid and the production of O_2^- in neutrophils exposed to Aroclor 1242 occurred with similar kinetics and concentration–response relations. Two inhibitors of phospholipase A_2, BPB and manoalide, caused concentration–dependent inhibition of O_2^- production upon exposure of rat neutrophils to Aroclor

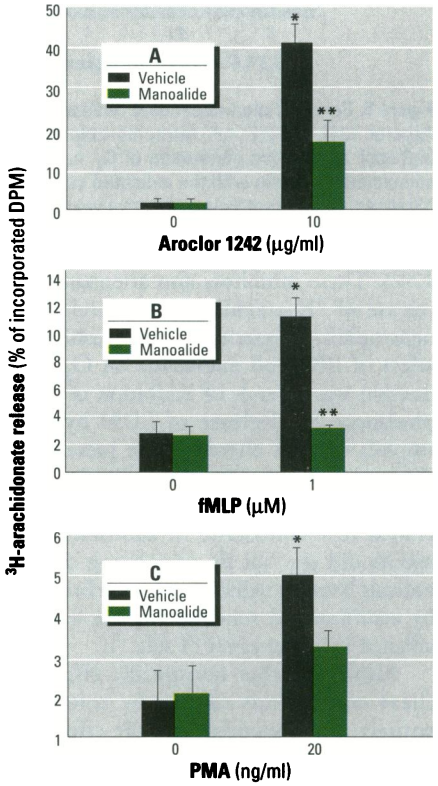

Figure 5. The inhibitory effect of manoalide on release of ^{3}H- arachidonic acid in neutrophils stimulated with (A) Aroclor 1242, (B) formyl-methionyl-leucyl-phenylalanine (fMLP), or (C) phorbol myristate acetate (PMA). Neutrophils were labeled with ^{3}H-arachidonic acid, preincubated with manoalide (10 μM) or vehicle for 20 min, then stimulated as described in Figure 1. Cumulative release of ^{3}H-arachidonate was measured over 20 min. Data are expressed as percent of total cellular radioactivity. *Statistically different from respective value obtained in the absence of stimulus. **Statistically different from respective value obtained in the absence of manoalide; $n = 4$.

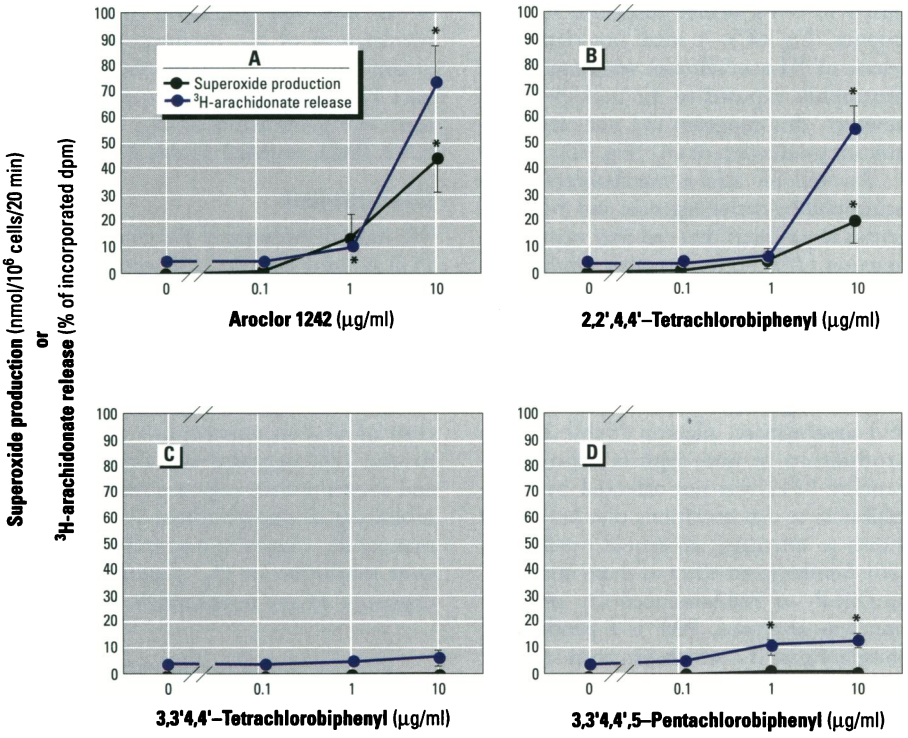

Figure 6. Concentration-dependent release of O_2^- and ^{3}H-arachidonate in neutrophils stimulated with (A) Aroclor 1242, (B) 2,2′,4,4′-tetrachlorobiphenyl (TCB), (C) 3,3′,4,4′-TCB, or (D) 3,3′,4,4′,5-pentachlorobiphenyl. Cumulative generation of O_2^- and release of ^{3}H-arachidonate were measured in neutrophils stimulated for 20 min with the indicated concentrations of PCBs, as described in Materials and Methods. *Statistically different from respective value obtained in the presence of vehicle; $n = 4$.

Table 2. Stimulated production of O_2^- in the presence of inhibitors of arachidonic acid metabolism and a platelet-activating factor receptor antagonist[a]

		Stimulus		
Addition	Concentration (μM)	Aroclor 1242 (10 μg/ml)	PMA (20 ng/ml)	fMLP (1 μM)
Aspirin	0	37 ± 1.3	18 ± 3.8	45 ± 7.6
	1	39 ± 3.1	19 ± 3.0	44 ± 4.0
	10	36 ± 3.2	18 ± 2.5	58 ± 3.4
	100	31 ± 1.4	17 ± 2.8	60 ± 2.1
Zileuton	0	33 ± 8	28 ± 4.4	15 ± 1.3
	0.1	41 ± 12	28 ± 7.4	20 ± 1.1
	1	42 ± 11	30 ± 8.0	19 ± 0.9
	10	30 ± 6.5	19 ± 4.2	19 ± 2.5
WEB 2086	0	48 ± 11	27 ± 11	22 ± 5.3
	1	47 ± 9.6	26 ± 11	21 ± 5.8
	10	45 ± 9.4	25 ± 12	20 ± 4.2
	100	37 ± 7.7	26 ± 9.3	18 ± 4.3

Abbreviations: PMA, phorbol myristate acetate; fMLP, formyl-methionyl-leucyl-phenylalanine.
[a]Neutrophils (2×10^6 cells/ml) were treated with aspirin, zileuton, WEB 2086, or vehicle as described in Materials and Methods. Cytochalasin B (4 μM) was added 5 min before stimulation with fMLP. No O_2^- was produced by unstimulated neutrophils either in the presence or absence of inhibitors; $n = 3$–5. No significant differences were observed.

1242. These inhibitors also attenuated the release of ^{3}H-arachidonate in prelabeled neutrophils, suggesting that the inhibitory effect of BPB and manoalide on O_2^- production was a result of inhibition of phospholipase A_2. Release of LDH by neutrophils was not altered in the presence of these compounds (Fig. 2), indicating that the inhibition of O_2^- generation was not related to cytotoxicity. In addition, these agents did not act by scavenging oxygen radicals because reduction of cytochrome C by xanthine and xanthine oxidase was not affected by either agent (Table 1).

Although other nonspecific effects of these compounds cannot be ruled out entirely, the argument that the effects on O_2^- production are due to inhibition of phospholipase A_2 is strengthened by the use of compounds that inhibit phospholipase A_2 by different mechanisms. Manoalide inhibits the enzyme by covalently modifying selective lysine residues within the protein (*21*), whereas BPB causes irreversible inhibition by binding covalently to a histidyl residue at the active site (*21*). Furthermore, quinacrine, which inhibits phospholipase A_2 by perturbing the enzyme–substrate interface (*21*), also decreased O_2^- measured in Aroclor 1242-treated neutrophils. The results with quinacrine, however, are difficult to interpret because concentrations that inhibited O_2^- production by neutrophils also inhibited the reduction of cytochrome C by O_2^- generated by xanthine and xanthine oxidase (Table 1), suggesting that quinacrine is capable of scavenging O_2^-. Thus, it is not possible to know from these data what contribution inhibition of phospholipase A_2 made to the decrease in O_2^- detected from activated neutrophils in the presence of quinacrine. These results suggest that studies using quinacrine to relate phospholipase A_2 activity to the production of oxygen radicals should be interpreted with caution.

Quinacrine is not the only inhibitor of phospholipase A_2 that has pleiotropic effects. In general, all inhibitors of phospholipase A_2 available at this time have been shown to have effects unrelated to the inhibition of phospholipase A_2 (*20,23-26*). Because of the lack of availability of an inhibitor specific for phospholipase A_2, it is difficult to make definitive conclusions concerning the role of this enzyme in cellular activation. Despite considerable efforts to minimize the probability that nonspecific effects of the inhibitors were responsible for our results, the possibility remains that inhibition of O_2^- production by manoalide and BPB occurred via other means. For example, the inhibition of O_2^- production in the presence of BPB and manoalide may have resulted from the ability of these agents to inhibit phosphoinositide-specific phospholipase C (*23,24*), as this enzyme has been shown to be involved in the activation of neutrophils by fMLP and Aroclor 1242 (*6*). However, this possibility seems unlikely because inhibition of phospholipase C occurs at higher concentrations of BPB [IC_{50} = 100 μM (*23*)] and manoalide [IC_{50} = 80 μM (*27*)] than the concentrations found to be effective in this study. Furthermore, BPB and manoalide inhibited the response to PMA, an agent that does not involve activation of phospholipase C (*28*), thus suggesting that the inhibitory effect of these compounds on O_2^- was unrelated to their ability to inhibit phospholipase C.

If activation of phospholipase A_2 is important in initiation of production of O_2^- in response to stimuli, release of ^{3}H-arachidonic acid from prelabeled neutrophils should precede generation of O_2^-. Significant release of ^{3}H-arachidonate was seen within 5 min in neutrophils exposed to Aroclor 1242 (Fig. 3A), and O_2^- production was observed between 5 and 15 min (*6*). Thus, the results are consistent with the hypothesis that phospholipase A_2 is involved in initiation of NADPH oxidase activity in neutrophils activated by Aroclor 1242.

Similar results were observed with fMLP: release of ^{3}H-arachidonate was seen within 2 min (Fig. 3B) and O_2^- was detected within 5 min (*6*). The concentration–

response relations for ^{3}H-arachidonate release and O_2^- production in neutrophils exposed to Aroclor 1242 (Fig. 5A) are also supportive of this hypothesis, as are most of the results from studies examining the effects of specific PCB congeners. For example, 2,2′,4,4′-TCB caused production of O_2^- and release of ^{3}H-arachidonate from neutrophils at a concentration of 10 µg/ml but not at 1 µg/ml. Conversely, 3,3′,4,4′-TCB failed to elicit either response. The exception was 3,3′,4,4′,5-PeCB, which like 3,3′,4,4′-TCB has high affinity for the Ah receptor. 3,3′,4,4′,5-PeCB caused small, but significant release of arachidonic acid in the absence of production of O_2^-. These results suggest that O_2^- generation by neutrophils exposed to PCBs is accompanied by the release of arachidonic acid, but this release is not sufficient to cause production of O_2^-. Alternatively, 3,3′,4,4′,5-PeCB may stimulate the release of arachidonic acid from a phospholipid pool that is different from that released in response to Aroclor 1242 and 2,2′,4,4′-TCB, and this pool may be unavailable for activation of the NADPH oxidase.

In rat neutrophils stimulated with PMA, it does not appear that activation of phospholipase A_2 is required for initiation of O_2^- generation. Release of ^{3}H-arachidonate was significant only after 20 min of exposure to the stimulus, while O_2^- production occurred much earlier (*29*). In addition, the magnitude of release of ^{3}H-arachidonate was considerably lower in neutrophils stimulated with PMA when compared to the results obtained with fMLP or Aroclor 1242, whereas the amount of O_2^- produced was comparable. One explanation for these data is that, although phospholipase A_2 is required for PMA-induced production of O_2^-, its role is not to initiate activation of NADPH oxidase. An alternative explanation for these results is that PMA activates phospholipase A_2 to release low concentrations of arachidonic acid that were not detectable by the methods employed in this study but that are necessary and sufficient in concert with other signal transduction pathways activated by PMA (e.g., protein kinase C) to stimulate production of O_2^-. This latter explanation is consistent with previous studies using human peripheral blood neutrophils in which O_2^- production in response to PMA was inhibited by BPB and quinacrine and was also abolished by staurosporine, an inhibitor of protein kinase C (*14*). The effect of staurosporine was likely due to inhibition of protein kinase C-mediated phosphorylation and translocation of the 47-kD cytosolic factor p47, an event that has been proposed to be required for assembly and activation of the NADPH oxidase (*30,31*). Thus, it appears that activation of the NADPH oxidase by PMA in human neutrophils requires at least two events: protein kinase C-mediated phosphorylation of p47 and phospholipase A_2-induced release of arachidonic acid. The same events may be required in rat neutrophils.

Although O_2^- production in response to Aroclor 1242 was abolished completely by 10 µM manoalide (Fig. 2B), release of ^{3}H-arachidonate was inhibited only partially (Fig. 4). There are at least three possible explanations for these results. One is that arachidonate release must reach a threshold to cause activation of the NADPH oxidase, and manoalide reduces arachidonic acid to levels below that threshold. The second is that, although the mechanism of release of arachidonic acid involves a phospholipase A_2 that can be inhibited by manoalide and BPB, other phospholipases A_2 may contribute to overall release of arachidonic acid. Several isoforms of phospholipase A_2 exist in neutrophils, all of which may not be inhibited by the compounds used in this study. For example, it is known that neutrophils contain a cytosolic phospholipase A_2 that is not a target for either manoalide or BPB (*20*).

A third possible explanation for the partial inhibition of arachidonate release by manoalide is that mechanisms independent of phospholipase A_2 are responsible, in part, for the release of arachidonic acid in stimulated neutrophils. One potential mechanism involves phospholipase D-dependent conversion of phoshatidylcholine or phosphatidylethanolamine to phosphatidic acid and subsequent release of arachidonic acid from diglyceride and monoglyceride by the actions of diglyceride lipase and monoglyceride lipase, respectively (*20*). Another potential source of arachidonic acid in activated neutrophils is diacylglycerol lipase-dependent release from diacylglycerol, which arises from membrane phosphoinositides as a consequence of phospholipase C activation (*32*). Although these pathways contribute less total arachidonic acid than phospholipase A_2 (*32*), they may be responsible for the arachidonate released in Aroclor 1242-activated neutrophils that was insensitive to manoalide. Indeed, in rat neutrophils exposed to PCBs, phospholipase C activity is increased (*6*), and diacylglycerol levels are likely to be elevated. Further studies are needed to address the role of these pathways in the release of arachidonic acid in neutrophils exposed to PCBs.

It is likely that arachidonic acid, rather than its metabolites, is responsible for stimulation of NADPH oxidase activity in neutrophils activated with Aroclor 1242. Inhibitors of cyclooxygenase and of 5-lipoxygenase as well as the PAF receptor antagonist WEB 2086 failed to alter O_2^- production in response to Aroclor 1242, fMLP, or PMA. These results are consistent with those obtained using other agents that stimulate neutrophils. Exposure of human neutrophils to exogenous arachidonate activates the NADPH oxidase (*33,34*), and this response is unaffected by inhibitors of the lipoxygenase or cyclooxygenase pathways. In addition, several other unsaturated fatty acids that are not substrates for metabolism via these pathways (e.g., linoleic, oleic, docosahexanoic, and eicosapentaenoic acid) also stimulate respiratory burst activity in intact neutrophils (*34,35*). Thus, it appears that phospholipase A_2-mediated release of arachidonic acid (or other unsaturated fatty acids) is an important event in activation of rat neutrophils by PCBs.

The role of arachidonic acid in activation of NADPH oxidase and consequent production of O_2^- has been reported in human peripheral blood neutrophils in response to soluble mediators (*12,14*) as well as particulate stimuli (*14*). To our knowledge, this is the first report demonstrating a role for phospholipase A_2 in activation of rat neutrophils. The results of the present study suggest that, in addition to PCBs, PMA and fMLP activate rat neutrophils by a mechanism similar to that seen in human peripheral blood neutrophils and that involves phospholipase A_2-dependent release of arachidonic acid. Furthermore, this response is an effect of arachidonic acid itself and not its metabolites.

In conclusion, the results presented in this study are consistent with the hypothesis that neutrophils, upon exposure to Aroclor 1242, produce O_2^- by a mechanism that involves phospholipase A_2-dependent release of arachidonic acid. The release of arachidonic acid by inflammatory cells upon exposure to PCBs may represent an additional mechanism of PCB-induced toxicity. Arachidonic acid and its metabolites have been implicated in a variety of inflammatory disease states including septicemia (*36,37*), rheumatoid arthritis (*38*), and systemic lupus erythematosus (*39*), as well as diseases characterized by cellular transformation such as hypertrophic transformation of the skin (*40*) and colon cancer (*41*). In addition, arachidonic acid has been implicated as a cytotoxicant in various models of tissue injury (*42,43*), and activation of phospholipase A_2 is required for the cytotoxic effects of cytokines such as tumor necrosis factor (*44*). Therefore, the activation of phospholipase A_2 and subsequent release of arachidonic acid from cells exposed to PCBs may be important in mechanisms of PCB toxicity.

References

1. Shain W, Bush B, Seegal R. Neurotoxicity of polychlorinated biphenyls:structure–activity relationship of individual congeners. Toxicol Appl Pharmacol 111:33–42 (1991).
2. Stahl BU, Beer DG, Weber LWD, Rozman K. Decreased hepatic phosphoenolpyruvate carboxykinase messenger ribonucleic acid (PEPCK-mRNA) and correspondingly reduced amounts of PEPCK protein after 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) treatment in rats. Toxicology 79:81–95 (1993).
3. Kodavanti PRS, Shafer TJ, Ward TR, Mundy WR, Freudenrich T, Harry GJ, Tilson HA. Differential effect of polychlorinated biphenyl congeners on phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar granule cells. Brain Res 662:75–82 (1994).
4. Kodavanti PRS, Ward TR, McKinney JD, Tilson HA. Increased [^{3}H] phorbol ester binding in rat cerebellar granule cells by polychlorinated biphenyl mixtures and congeners: structure–activity relationships. Toxicol Appl Pharmacol 130:140–148 (1995).
5. Ganey PE, Sirois JE, Denison M, Robinson JP, Roth RA. Neutrophil function after exposure to polychlorinated biphenyls *in vitro*. Environ Health Perspect 101:430–434 (1993).
6. Tithof PK, Contreras ML, Ganey PE. Aroclor 1242 stimulates the production of inositol phosphates in polymorphonuclear leukocytes. Toxicol Appl Pharmacol 131:136–143 (1995).
7. Brown AP, Ganey PE. Neutrophil degranulation and superoxide production induced by polychlorinated biphenyls are calcium dependent. Toxicol Appl Pharmacol 131:198–205 (1995).
8. Cockcroft S, Bennett JP, Gomperts BD. The dependence on Ca^{2+} of phosphatidylinositol breakdown and enzyme secretion in rabbit neutrophils stimulated by formylmethionyl-leucyl-phenylalanine or ionomycin. Biochem J 200:501–508 (1981).
9. Rossi F, Bianca VD, Brzeskowiak M, DeTogni P, Cabrini G. Relationships between phosphoinositide metabolism, Ca^{2+} changes and respiratory burst in formyl-methionyl-leucyl-phenylalanine-stimulated human neutrophils. FEBS Lett 181:253–258 (1985).
10. Crockett-Torabi E, Fantone JC. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fc receptor-specific mechanisms. J Immunol 145:3026–3032 (1990).
11. Cockcroft S. G-protein-regulated phospholipases C, D and A_2-mediated signalling in neutrophils. Biochim Biophys Acta 1113:135–160 (1992).
12. Neal TM, Vissers MCM, Winterbourne CC. Inhibition by nonsteroidal anti-inflammatory drugs of superoxide production and granule enzyme release by polymorphonuclear leukocytes stimulated with immune complexes or formyl-methionyl-leucyl-phenylalanine. Biochem Pharmacol 36:2511–2517 (1987).
13. Henderson LM, Moule SK, Chappell JB. The immediate activator of the NADPH oxidase is arachidonate not phosphorylation. Eur J Biochem 211:157–162 (1993).
14. Dana R, Malech HL, Levy R. The requirement for phospholipase A_2 for activation of the assembled NADPH oxidase in human neutrophils. Biochem J 297:217–223 (1994).
15. Walker BAM, Seiler AJ, Owens CA, Hagenlocker BE, Ward PA. Absence of fMLP receptors on rat macrophages. J Leukocyte Biol 50:600–606 (1991).
16. Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52:741–744 (1973).
17. Bergmeyer HV, Bernt E. Lactate dehydrogenase UV assay with pyruvate and NADH. In: Methods of enzymatic analysis (Bergmeyer HV, ed). New York:Academic Press, 1974;524–579.
18. Smith DM, Waite M. Phosphatidylinositol hydrolysis by phospholipase A_2 and C activities in human peripheral blood neutrophils. J Leukocyte Biol 52:670–678 (1992).
19. Sandborg RR, Smolen JE. Biology of disease. Early biochemical events in leukocyte activation. Lab Invest 59:300–320 (1988).
20. Smith WL. Prostanoid biosynthesis and mechanisms of action. Am J Physiol 263:F181–F191 (1992).
21. Symer DE, Paznekas WA, Shin HS. A requirement for membrane-associated phospholipase A_2 in platelet cytotoxicity activated by receptors for immunoglobulin G and complement. J Exp Med 177:937–947 (1993).
22. Stewart AG, Harris T. Platelet-activating factor may participate in signal transduction processes in rabbit leukocytes. Lipids 26:1044–1049 (1991).
23. Best L, Sener A, Mathias PCF, Malaisse WJ. Inhibition by mepacrine and p-bromophenacylbromide of phosphoinositide hydrolysis, glucose oxidation, calcium uptake and insulin release in rat pancreatic islets. Biochem Pharmacol 33:2657–2662 (1984).
24. Bennett CF, Mong S, Wu HW, Clark MA, Wheeler L, Crooke ST. Inhibition of phosphoinositide-specific phospholipase C by manoalide. Mol Pharmacol 32:587–593 (1987).
25. Wheeler LA, Sachs G, DeVries G, Goodrum D, Woldemussie E, Muallem S. Manoalide, a natural sesterterpenoid that inhibits calcium channels. J Biol Chem 262:6531–6538 (1987).
26. Lister MD, Glaser KB, Ulevitch RJ, Dennis EA. Inhibition studies on the membrane-associated phospholipase A_2 in vitro and prostaglandin E_2 production in vivo of the macrophage-like $P388D_1$ cell. Effects of manoalide, 7,7-dimethyl-5,8-eicosadienoic acid, and p-bromophenacyl bromide. J Biol Chem 264:8520–8528 (1989).
27. De Vries GW, Amdahl LD, Kramer KD, Wheeler LA. Inhibition by manoalide of fMLP-stimulated elastase release from human neutrophils. Biochem Pharmacol 40:2487–2494 (1990).
28. Tauber AI. Protein kinase C and the activation of the human neutrophil NADPH-oxidase. Blood 69:711–720 (1987).
29. Dahm LJ, Hewett JA, Roth RA. Bile and bile salts potentiate superoxide anion release from activated, rat peritoneal neutrophils. Toxicol Appl Pharmacol 95:82–92 (1988).
30. Nauseef WM, Volpp BD, McCormick S, Leidal KG, Clark RA. Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. J Biol Chem 266:5911–5917 (1991).
31. Dusi S, Rossi R. Activation of NADPH oxidase of human neutrophils involves the phosphorylation and the translocation of cytosolic p67 phox. Biochem J 296:367–371 (1993).
32. Sha'afi RI, Molski TFP. Activation of the neutrophil. Prog Allergy 42:1–64 (1988).
33. Yamaguchi T, Kaneda M, Kakinuma K. Effect of saturated and unsaturated fatty acids on the oxidative metabolism of human neutrophils. The role of calcium ion in the extracellular medium. Biochem Biophys Res Commun 861:400–404 (1986).
34. Poulos A, Robinson BS, Ferrante A, Harvey DP, Hardy SJ, Murray AW. Effect of 22-32 carbon n-3 polyunsaturated fatty acids on superoxide production in human neutrophils: synergism of docosahexaenoic acid with f-met-leu-phe and phorbol ester. Immunology 73:102–108 (1991).
35. Hardy SJ, Ferrante A, Poulos A, Robinson BS, Johnson DW, Murray AW. Effect of exogenous fatty acids with greater than 22 carbon atoms (very long chain fatty acids) on superoxide production by human neutrophils. J Immunol 153:1754–1761 (1994).
36. Green JA, Smith GM, Buchta R. Circulating phospholipase A_2 activity associated with sepsis and septic shock is indistinguishable from that associated with rheumatoid arthritis. Inflammation 15:355–367 (1991).
37. Vadas P, Hay JB. Involvement of circulating phospholipase A_2 in the pathogenesis of the hemodynamic changes in endotoxin shock. Can J Physiol Pharmacol 61:561–566 (1983).
38. Pruzanski W, Vadas P, Stefanski E, Urowitz M. Phospholipase A_2 activity in sera and synovial fluids in rheumatoid arthritis and osteoarthritis. Its possible role as a proinflammatory enzyme. J Rheumatol 12:211–216 (1985).
39. Pruzanski W, Goulding NJ, Flower RJ, Glandman DD, Urowitz MB, Goodman PJ, Scott KF, Vadas P. Circulating group II phospholipase A_2 activity and anitlipocortin antibodies in systemic lupus erythematosus. Correlative study with disease activity. J Rheumatol 21:252–257 (1994).
40. Marks F, Furstenberger G. Proliferative responses of the skin to external stimuli. Environ Health Perspect 101(Suppl 5):95–101 (1993).
41. Rigas B, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. J Lab Clin Med 122:518–523 (1993).
42. Cifone MG, Botti D, Festuccia C, Napolitano T, Del Grosso E, Cavallo G, Chessa MA, Santoni A. Involvement of phospholipase A_2 activation and arachidonic acid metabolism in the cytotoxic functions of rat NK cells. Cell Immunol 148:247–258 (1993).
43. Takenaka K, Kassall NF, Foley PL, Lee KS. Oxyhemoglobin-induced cytotoxicity and arachidonic acid release in cultured bovine endothelial cells. Stroke 24:839–845 (1993).
44. Suffys P, Beyaert R, De Valck D, Vanhaesebroeck B, Van Roy F, Fiers W. Tumour necrosis factor-mediated cytotoxicity is correlated with phospholipase A_2 activity, but not with arachidonic acid release per se. Eur J Biochem 195:465–475 (1991).